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Endogenous RNase inhibitor contributes to stability of RNA in crude cell lysates: Applicability to RT-qPCR

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ABSTRACT

Crude cell lysates are increasingly used as input for direct analysis by reverse transcription quantitative PCR (RT-qPCR), particularly for high-throughput applications. We previously demonstrated that a simple buffer containing a non-ionic detergent can serve as an inexpensive alternative to commercial cell-lysis reagents for the preparation of RT-qPCR-ready cell lysates; addition of an exogenous RNase inhibitor (RI) to the lysis buffer was found to be unnecessary to maintain RNA stability in cell lysates either freshly prepared or previously stored frozen at −80 °C. In the present study, we have demonstrated that the stability of RNA observed in our cell lysates is due to the presence of the endogenous RI. Furthermore, we have established the generalizability and applicability of this phenomenon by evaluating lysates prepared from cell lines commonly used in virology (A549, HeLa, MDCK, and Vero). Awareness of the mechanism underlying RNA stability may engender greater confidence in generating cell lysates for RT-qPCR without relying on addition of exogenous RI (a substantial cost-saving benefit) and encourage appropriate practices for handling and storage of samples.

1. Introduction

Crude cell lysates are increasingly used as input for reverse transcription quantitative PCR (RT-qPCR). Several commercial cell-lysis reagents for this purpose are available (such as Ambion Cells-to-C_T and Bio-Rad iScript Sample Preparation Reagent). Ease of use contributes to the appeal of these reagents, particularly for high-throughput applications in which conventional nucleic acid extraction/purification from samples would be cumbersome and rate-limiting. Recent studies suggest that crude cell lysates may offer comparable accuracy [1,2] and superior sensitivity [1] for RT-qPCR compared with purified RNA.

In order to mitigate the cost constraint imposed by commercial reagents, we developed a simple cell-lysis buffer (CL Buffer) containing a non-ionic detergent (0.25% Igepal CA-630) as an alternative and demonstrated its feasibility for generating RT-qPCR-ready cell lysates [3]. Experimental samples from adherent cells are prepared in straightforward steps by (1) washing cells, (2) briefly exposing cells to CL Buffer at room temperature, and (3) collecting the resulting cell lysates for frozen storage or immediate analysis by RT-qPCR. We found that addition of commercial RNase inhibitor (RI) to CL Buffer was not necessary to maintain RNA integrity in cell lysates either freshly prepared or previously stored frozen at −80 °C. Avoiding the requirement to add commercial RI would be a notable cost-saving benefit if it were routinely possible.

In the present study, we have established that the maintenance of RNA integrity in our cell lysates is due to the presence of endogenous RI activity. We have verified the generalizability of this phenomenon by evaluating lysates prepared from four cell lines commonly used in virology (A549, HeLa, MDCK, and Vero). In addition, we have successfully demonstrated applicability to RT-qPCR, in a manner similar to our previous studies [4,5], using lysates prepared from cells infected with influenza virus or respiratory syncytial virus (RSV). Mechanistic insights might foster confidence in data quality derived from this experimental approach and encourage appropriate procedures for handling of cell-lysate samples.

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2. Materials and methods

2.1. Cells and viruses

MDCK cells (London line) [6] were obtained from J. Weir (Division of Viral Products, OVRR, CBER, FDA). A549, HeLa, and Vero cells were obtained from the American Type Culture Collection. All cells were propagated using Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum (Hyclone) and 2 mM glutamine. Influenza virus A/PR/8/34 and RSV B1 were obtained from Z. Ye and J. Beeler, respectively (Division of Viral Products, OVRR, CBER, FDA). Infectivity was determined by calculating the 50% tissue culture infective dose ($TCID_{50}$) by titration using MDCK cells (for influenza virus) and Vero cells (for RSV).

2.2. Preparation of cell lysates

Cells were seeded in 10-cm culture dishes (4×10^6 cells per dish). On the following day, cells were washed twice with phosphate-buffered saline (PBS) and then exposed to CL Buffer (6 mL per dish; 10 mM Tris-HCl pH 7.4, 0.25% Igepal CA-630, 150 mM NaCl) with or without 1 mM dithiothreitol (DTT; Thermo Scientific; 20291) for 5 min at room temperature (~22 °C). The resulting lysates were collected and used immediately or stored frozen at the temperature indicated for the experiment. Cell-exposure time to CL Buffer was shortened to 2 min for some experiments to prevent non-adherence occasionally observed for one of the cell types (Vero). The soluble fraction was obtained either by (1) recovering the supernatant (UC supernatant) after subjecting the cell lysate to ultracentrifugation (208,000 × g) for 4 h at 4 °C using an SW41 rotor (modification of a protocol described by Rio et al. [7]), or (2) recovering the filtrate (Amicon filtrate) after passing the cell lysate through an Amicon Ultra filtration device (EMD Millipore; molecular weight cutoff of 100,000) by centrifugation (4000 × g) for 30 min at 4 °C. For certain experiments involving lysates, the following additions were used: RNasin Plus (Promega; final concentration of 1 unit/μL), a monoclonal antibody (Ab) specific to mammalian RI (reactive across mammalian species including dog and human; clone 3F11; TA501875; Origene; 1 μg per 200 μL lysate), and a monoclonal Ab specific to glyceraldehyde-3-phosphate dehydrogenase (GAPDH; clone 2D9; TA802519; Origene; 1 μg per 200 μL lysate).

2.3. Western blot analysis

Cell lysates were prepared using a non-ionic detergent lysis buffer (50 mM Tris-HCl pH 8.0, 1% Igepal CA-630, and 150 mM NaCl) supplemented with a protease inhibitor cocktail (P8340; Sigma; 1:100 dilution). Protein concentration was determined using the Pierce BCA kit (Thermo Fisher Scientific).

Cell lysates (12 μg per lane) were applied to NuPAGE Bis-Tris 4–12% gels (Life Technologies), and electrophoresis was performed under reducing conditions using MOPS SDS Running Buffer (Life Technologies). Proteins were transferred to PVDF membranes. After blocking with 5% non-fat milk, PVDF membranes were incubated with primary Ab against mammalian RI (TA501875; Origene; 1:500) or β-actin (3700; Cell Signaling Technology; 1:1000). Following incubation with an HRP-conjugated secondary Ab against mouse IgG, protein bands were visualized by chemiluminescence using the Amersham ECL system (GE Healthcare Life Sciences).

2.4. Microfluidics-based analysis of RNA

Total RNA was purified from cell lysates (~200 μL) using the RNeasy Mini kit (Qiagen) according to the "cleanup" protocol supplied with the kit. RNA was eluted in 30 μL of nuclease-free water and stored frozen at −80 °C until assessment. Samples (1 μL) were subjected to microfluidics-based Experion RNA StdSens electrophoresis (Bio-Rad). RNA Quality Indicator (RQI) values were calculated in an automated and unbiased manner (by an algorithm described in Bio-Rad Technical Note 5761) using the Experion software version 3.2. According to the default Experion setting, RQIs between 7.0 and 10.0 indicate RNA of acceptable quality for most downstream applications.

2.5. RT-qPCR analysis

A549, HeLa, MDCK, and Vero cells were seeded in 96-well culture plates (15,000 cells per well). On the following day, cells were infected with a dilution series (3-fold) of either influenza virus strain A/PR/8/34 or RSV strain B1 ($TCID_{50}$ per well ranging from 100 to 8100). Infected cells (at 6 h post-infection for influenza virus and 24 h post-infection for RSV) were washed twice with PBS (100 μL per well) and then exposed to CL Buffer containing 1 mM DTT (100 μL per well) for 2 min at room temperature (~22 °C). The resulting lysates were subjected to RT-qPCR analysis.

RT-qPCR experimental design was facilitated by awareness of the MIQE guidelines [8]. RT-qPCR was performed as described in earlier studies [4,5]. For RT-qPCR targeting the influenza virus A matrix-gene transcript, the following PCR primers were used: AAGACCAATCCTGTCACCTCTGA and CAAAGCGTCTACGCTGCAGTCC. Each reaction for influenza virus contained: 1 μL of cell lysate, 1X iScript One-Step SYBR Green RT-PCR Supermix (170–8893; Bio-Rad), 600 nM of each primer (synthesized at the Facility for Biotechnology Resources; CBER, FDA; Silver Spring, MD), 0.2 μL iScript reverse transcriptase, and nuclease-free water to 10 μL. A CFX96 real-time PCR instrument (Bio-Rad) was used with the following protocol: 50 °C for 10 min (1X), 95 °C for 5 min (1X), 95 °C for 10 s/61 °C for 15 s/72 °C for 30 s (40X; data collection occurred after the 72 °C step). For RT-qPCR targeting the RSV N gene transcript (subgroup B), the following PCR primers were used: CTGTCATCCAGCAAATACACTATTCA and GCACATCATAATTGGGAGTGTCA. Each reaction for RSV contained: 1 μL of cell lysate, 1X iScript One-Step SYBR Green RT-PCR Supermix, 300 nM of each primer, 0.2 μL iScript reverse transcriptase, and nuclease-free water to 10 μL. The following thermocycling protocol was used: 50 °C for 10 min (1X), 95 °C for 5 min (1X), 95 °C for 10 s/64 °C for 30 s (40X; data collection occurred after the 64 °C step). Total RNA standards purified from cells infected with influenza virus or RSV were used as described previously [4,5]. For each RT-qPCR run, a 10-fold dilution series of RNA standard (using a cell lysate prepared from uninfected cells as the diluent) was assessed to verify RT-qPCR performance and to facilitate quantification. In addition, each RT-qPCR run included negative controls (lysate from uninfected cells as input) and no-reverse transcription controls (initial dilution of RNA standard described above as input); these controls typically result in no amplification, or occasionally, low-level non-specific amplifications (indicated by melt-curve analysis) with quantification cycle (C_q) values > 36.

3. Results

3.1. Impact of DTT on RNA stability of cell lysates following frozen storage at −20 °C and stress at 37 °C

We previously demonstrated the feasibility of using our CL Buffer (10 mM Tris-HCl pH 7.4, 0.25% Igepal CA-630, and 150 mM NaCl) for generating RT-qPCR-ready cell lysates using MDCK cells [3]. We hypothesized that the stability of RNA observed in cell

lysates either freshly prepared or following frozen storage at −80 °C, but not observed following frozen storage at −20 °C, may be due to the presence of the endogenous RI. Mammalian RI is a cytosolic protein rich in cysteine residues and requires reducing agents such as DTT for long-term maintenance of its activity under storage [9,10]. We evaluated RNA stability of cell lysates prepared using CL Buffer with or without 1 mM DTT. MDCK cells were exposed to CL Buffer (+/− DTT) for 5 min at room temperature (~22 °C). The resulting lysates were portioned into aliquots and stored frozen at −20 °C for up to 15 days. After frozen storage, aliquots were thawed and subjected to incubation for 1 h either on ice or at 37 °C. Immediately after incubation, total RNA was column-purified (Qiagen RNeasy) and subjected to microfluidics-based electrophoresis (Bio-Rad Experion). The virtual gel and associated RQIs (ranging from 1.0 for degraded RNA to 10.0 for intact RNA) for this experiment are shown in Fig. 1A. For lysate samples prepared with CL Buffer without DTT, RNA integrity was maintained after thermal stress following 1 day of frozen storage at −20 °C (RQIs of 10.0 and 9.3 for samples incubated on ice or at 37 °C, respectively; Fig. 1A, lanes 1 and 2); however, after 7 days of frozen storage, RNA became susceptible to degradation during thermal stress (RQIs of 9.0 and 2.9 for samples incubated on ice or at 37 °C, respectively; Fig. 1A, lanes 3 and 4). The results obtained in the absence of DTT are similar to those obtained in our earlier study [3]. For lysate samples prepared with CL Buffer containing DTT, the transition for RNA susceptibility was delayed; RNA quality was still acceptable after thermal stress following 7 days of frozen storage (RQIs of 9.7 and 8.7 for samples incubated on ice or at 37 °C, respectively; Fig. 1A, lanes 9 and 10). These data are consistent with DTT facilitating the preservation of endogenous RI activity. However, DTT

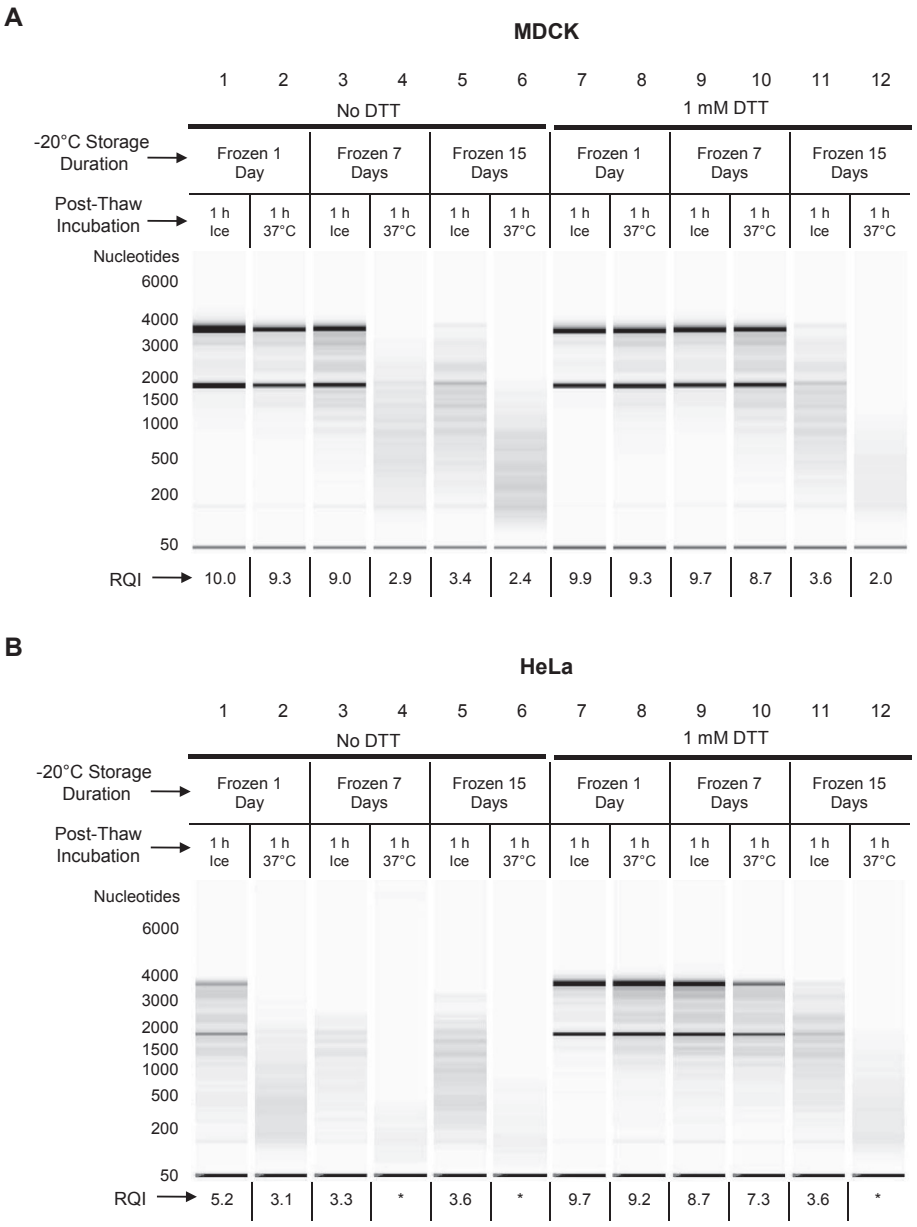

Fig. 1. *Impact of DTT on RNA stability of cell lysates.* (**A**) Cell lysates were prepared from MDCK cells by exposure to CL Buffer without (lanes 1–6) or with 1 mM DTT (lanes 7–12). Aliquots were stored frozen at −20 °C. On the indicated day, samples (200 μL) were thawed and subjected to the specified incubation. Immediately following incubation, RNA was purified from samples and subjected to Experion electrophoresis. The virtual gel and associated RQIs are shown. (**B**) A similar experiment as shown in (A) was performed using cell lysates prepared from HeLa cells. An asterisk (*) indicates that RNA concentration was too low for calculation of RQI (<5 ng/μL).

addition alone was insufficient to maintain RNA integrity after long-term sample storage at −20 °C, since 15 days of frozen storage at −20 °C resulted in extensive RNA degradation with RQIs of 3.6 (ice incubation; Fig. 1A, lane 11) and 2.0 (37 °C incubation; Fig. 1A, lane 12).

A similar experiment was performed using lysates prepared with HeLa cells (Fig. 1B). The impact of DTT addition was more evident with HeLa cell lysates compared with MDCK cell lysates. In the absence of DTT, RNA degradation was observed even for samples stored at −20 °C for only 1 day (RQIs of 5.2 and 3.1 for samples incubated on ice or at 37 °C, respectively; Fig. 1B, lanes 1 and 2). In the presence of DTT, RNA quality was preserved for samples stored at −20 °C for up to 7 days (Fig. 1B, lanes 7–10; RQIs ≥7.3); however, long-term sample storage at −20 °C (15 days) was still problematic despite DTT addition (Fig. 1B, lanes 11 and 12).

3.2. Association of RNase-inhibitor activity with the soluble fraction of cell lysates

In order to gain insight on the mechanism of RNA stability observed in our crude cell lysates, a fractionation experiment was performed. Cell lysate was freshly prepared from MDCK cells (by exposure to CL Buffer without DTT). The soluble fraction was obtained either by (1) recovering the supernatant (UC supernatant) after subjecting the cell lysate to ultracentrifugation (208,000 × g; 4 h), or (2) recovering the filtrate (Amicon filtrate) after passing the cell lysate through an Amicon Ultra filtration device (molecular weight cutoff of 100,000). These procedures also effectively removed high molecular weight ribosomal complexes and associated ribosomal RNA (which typically accounts for more than 90% of total RNA). The protocol for RNA column purification was applied to samples of UC supernatant and Amicon filtrate; Experion electrophoresis verified the efficient removal of RNA (Fig. 2, lanes 1 and 2). In order to localize the activity responsible for conferring RNA stability, MDCK cell lysate (prepared with CL Buffer without DTT and previously stored frozen for 7 days at −20 °C) was mixed with equal volume of UC supernatant, Amicon filtrate, CL Buffer (negative control), or CL Buffer containing a commercial RI (positive control); MDCK lysate primed by −20 °C storage in this manner was a convenient choice for this experiment due to its property shown in Fig. 1A (*i.e.*, RNA was consistently intact, but succumbed to degradation following 37 °C stress for 1 h). After incubation for 1 h either on ice or at 37 °C, RNA was purified from the mixtures and subjected to Experion electrophoresis (RNA present in UC supernatant/Amicon filtrate was minimal and did not confound this analysis). Mixing with CL Buffer did not prevent RNA degradation following thermal stress (Fig. 2, lanes 3 and 4); RQIs were 8.6 (ice) and 2.7 (37 °C), consistent with previous data (Fig. 1A). However, both UC supernatant (Fig. 2, lanes 5 and 6) and Amicon filtrate (Fig. 2, lanes 7 and 8) prevented RNA degradation to a degree comparable with CL Buffer containing RI (RNasin Plus, final concentration of 1 unit/μL; Fig. 2, lanes 9 and 10); RQIs following 37 °C incubation were 7.3 (UC supernatant), 8.0 (Amicon filtrate), and 8.4 (CL Buffer + RNasin Plus). The data suggest that the RNA degradation occurring under our experimental conditions is largely due to nucleases sensitive to inhibition by exogenous RI. Furthermore, the association of RI-like activity with the soluble fraction is consistent with the known cytoplasmic localization and size (molecular weight of ~50,000) of mammalian RI [9].

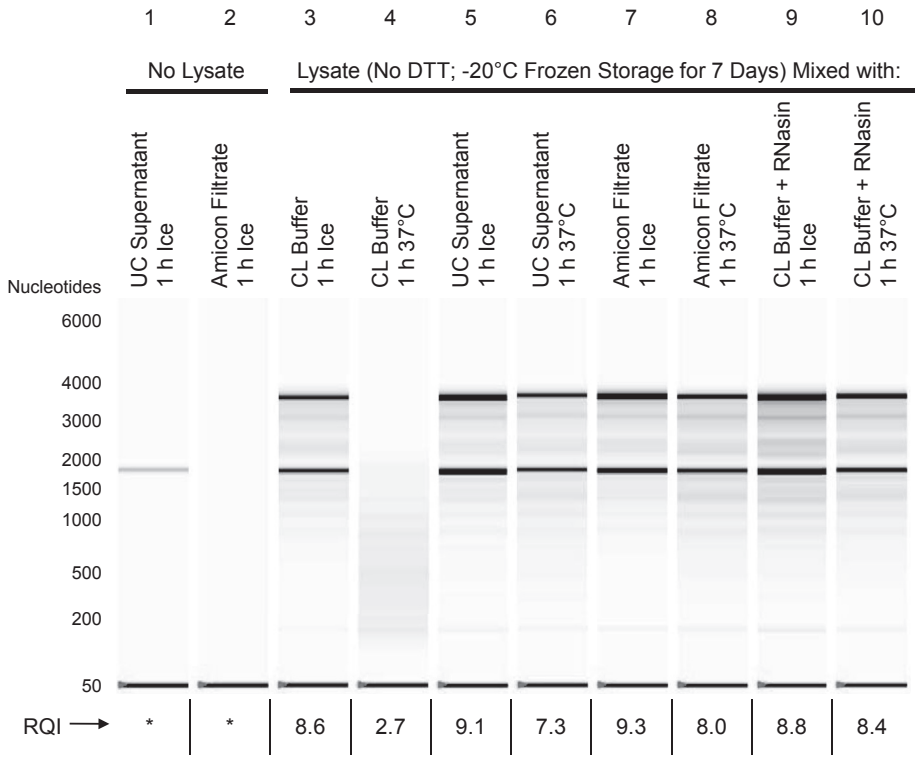

Fig. 2. *Association of RI activity with the soluble fraction.* Cell lysate was freshly prepared from MDCK cells by exposure to CL Buffer (without DTT), and the soluble fraction was obtained by two methods (UC supernatant and Amicon filtrate). RNA was purified from 200 μL of UC supernatant (lane 1) and Amicon filtrate (lane 2) and subjected to Experion analysis. MDCK lysate (100 μL; prepared from CL Buffer without DTT and previously stored frozen at −20 °C for 7 days) was mixed with equal volume of UC supernatant (lanes 5 and 6), Amicon filtrate (lanes 7 and 8), CL Buffer (lanes 3 and 4), or CL Buffer containing RNasin Plus (final concentration of 1 unit/μL; lanes 9 and 10); after the specified incubation, RNA was purified from the mixtures and subjected to Experion analysis. An asterisk (*) indicates that RNA concentration was too low for calculation of RQI (<5 ng/μL).

3.3. Endogenous RI contributes to RNA stability in lysates prepared from cell lines used in virology

We assessed the expression of endogenous RI by Western blot analysis. In addition to MDCK cells, we evaluated three cell lines commonly used in virology: A549 (human lung adenocarcinoma), HeLa (human cervical carcinoma), and Vero (African green monkey kidney). Detectable expression of RI was observed in all cell lines tested (Fig. 3), consistent with data provided by the commercial supplier that the monoclonal Ab used for Western blotting (3F11) is capable of recognizing RI from diverse species (including dog and primates).

Cell lysates were freshly prepared from A549, HeLa, Vero, and MDCK cells by exposure to CL Buffer (in the presence of 1 mM DTT). Monoclonal Ab specific to either RI (3F11) or an irrelevant target (GAPDH) was added to lysate samples (1 μg per 200 μL lysate). Prior to this lysate experiment, the level of contaminating RNase activity associated with each Ab reagent was assessed in experiments using purified RNA. Both the RI-specific Ab and the control GAPDH Ab were associated with detectable RNase activity (reinforcing its ubiquity in materials deriving from biological sources); however, the extent of RNase contamination appeared to be comparable between the two Ab reagents (Fig. 1 in Ref. [11]). In addition, the ability of this RI-specific Ab to interfere with the function of RI was demonstrated using purified components (Fig. 2 in Ref. [11]). Following addition of Ab, lysate samples were incubated for 1 h either on ice or at 37 °C; RNA was then purified and subjected to Experion analysis. Addition of RI-specific Ab led to extensive RNA degradation following thermal stress (Fig. 4, lanes 3, 6, 9, and 12). In contrast, addition of control GAPDH Ab allowed RNA stability to be maintained following thermal stress (Fig. 4, lanes 2, 5, 8, and 11) with RQIs ≥9.1. Overall, the data suggest that the RI-specific Ab used in this experiment is capable of inhibiting the function of endogenous RI in crude cell lysates, thereby leading to RNA degradation. Comparability of minor contaminating RNase activity associated with the Ab reagents argues against confounding stemming from this source.

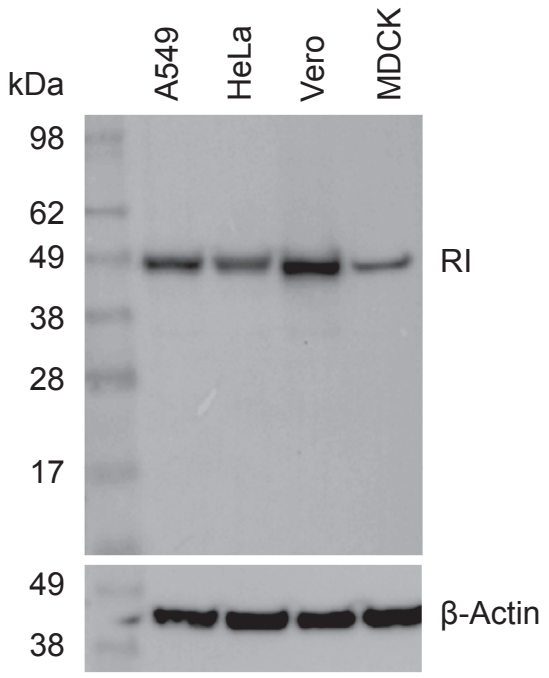

Fig. 3. *Expression of endogenous RI in cell lines*. Whole-cell lysates from A549, HeLa, Vero, and MDCK cells were subjected to Western blot analysis using primary mouse monoclonal Ab specific to either RI (3F11) or β-actin.

3.4. Applicability of crude cell lysates to downstream RT-qPCR

The applicability of our approach to RT-qPCR was explored using cells besides MDCK cells. A549, HeLa, Vero, and MDCK cells were infected with dilution series (3-fold) of either influenza virus strain A/PR/8/34 or RSV strain B1 using an input per well ranging from 100 to 8100 $TCID_{50}$ (in a manner similar to our previous studies [4,5]). Cell lysates were generated from infected cells using CL Buffer (with 1 mM DTT) and subjected to one-step SYBR Green RT-qPCR targeting virus gene transcripts (influenza virus M1 or RSV N). It is important to note that influenza virus and RSV have no DNA intermediates in their respective life cycle. Graphs in which C_q is plotted against $\log_{10}(TCID_{50})$ are shown for data generated with influenza virus (Fig. 5A) and RSV (Fig. 5B). Acceptable linearity (and by inference, accuracy in relative terms) was observed for both viruses across the tested range of input ($r^2 \geq 0.968$).

4. Discussion

We have established that the stability of RNA in crude cell lysates generated using our CL Buffer is due to the presence of endogenous RI as opposed to competing explanations (including the absence of RNases in cell lysates or the resistance of native RNA complexes in cell lysates to degradation by RNases). We have arrived at this conclusion on the basis of three corroborating lines of evidence. First, improved resistance to RNA degradation was observed in cell lysates generated with CL Buffer containing 1 mM DTT and stored at −20 °C (Fig. 1A and B), consistent with the known requirement of RI for reducing agents for optimal maintenance of activity [9]. A direct inhibitory effect of 1 mM DTT on RNase activity is unlikely to be the primary cause of RNA stability under our experimental conditions (as demonstrated in Fig. 2 of Ref. [11]); furthermore, a study of purified bovine pancreatic RNase A demonstrated that denaturation by 30 mM DTT (30-fold molar excess over RNase A) occurs over several hours at 30 °C [12]. Second, an RI-like activity was found to be associated with the soluble fraction of MDCK cell lysates (Fig. 2), consistent with the known cytosolic localization of RI [9]. Finally, enhanced susceptibility to RNA degradation was observed in cell lysates containing a monoclonal Ab specific for RI (Fig. 4). It is apparent that the extraordinary affinity of RI for target ribonucleases (dissociation constant, K_d, in the femtomolar range, comparable with avidin-biotin) [9,10] allows its inhibitory effect to manifest despite the extensive dilution experienced by cytosolic components in our cell lysates.

If the presence of endogenous RI in samples were to allow the avoidance of adding exogenous RI from a commercial source, significant cost-saving would be realized. The logistical impact would be magnified for high-throughput applications for which a premium is usually placed on minimizing per-sample cost. The Bio-Rad iScript Sample Preparation Reagent, the commercial cell-lysis reagent with which we have the most experience [4,5], costs ~$0.60 per 100 μL sample. Addition of RNasin Plus to a lysis buffer would cost ~$3 per 100 μL sample at the recommended concentration of 1 unit/μL, or ~$0.30 per 100 μL sample at 1/10 of the recommended concentration. For CL Buffer without addition of commercial RI, the per-sample cost for generating cell lysates is essentially negligible. Reagents that can serve as alternatives to protein-based inhibitors of RNases have recently become available. RNA*secure* (Life Technologies) irreversibly inactivates RNases in solution likely through a mechanism mediated by a combination of heat (60 °C for 10 min) and reducing activity. RNA*secure*, added as a

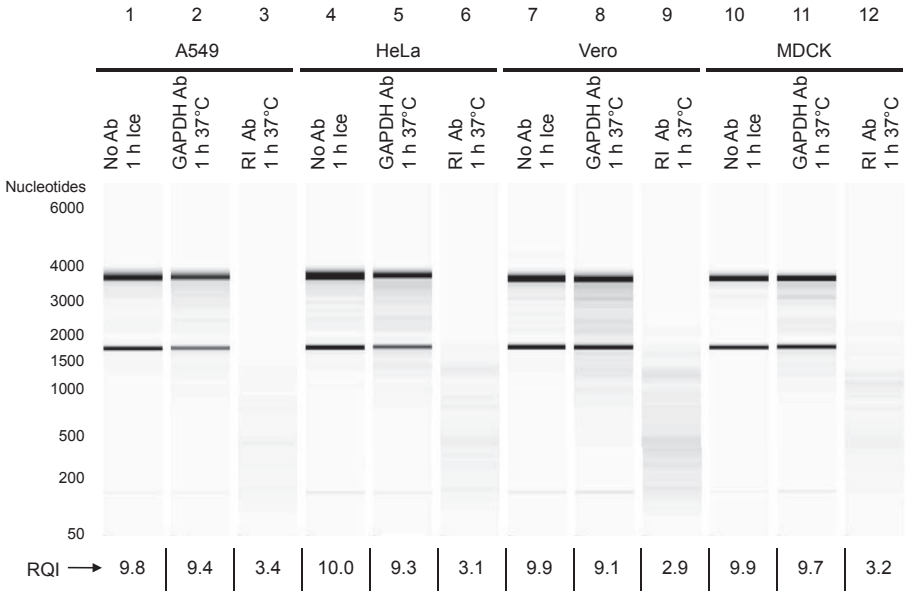

Fig. 4. *Impact of RI-specific Ab on RNA stability of cell lysates.* Cell lysates freshly prepared with CL Buffer supplemented with 1 mM DTT were mixed with monoclonal Ab (1 μg per 200 μL lysate) specific to either RI (3F11) or GAPDH. Following the specified incubation, RNA was purified and subjected to Experion analysis.

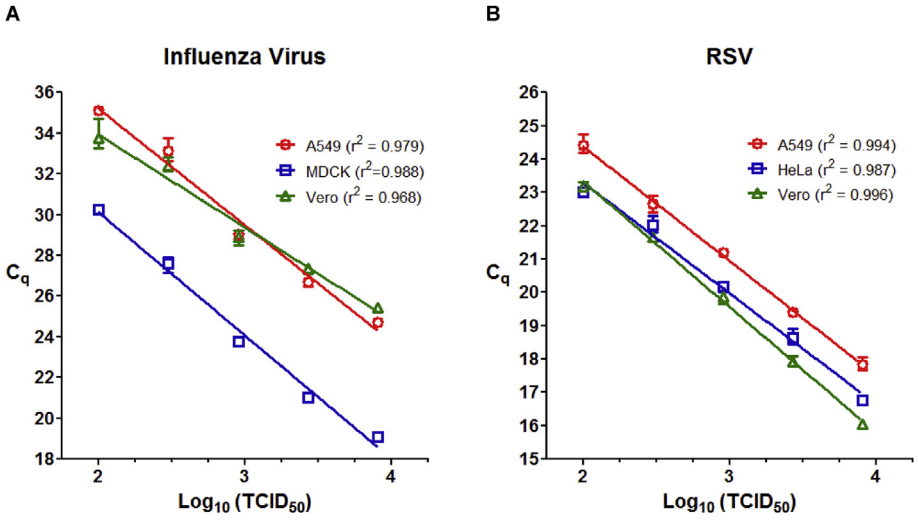

Fig. 5. *Applicability of crude cell lysates (A549, HeLa, MDCK, and Vero) to downstream RT-qPCR.* A549, HeLa, Vero, and MDCK cells were infected with dilution series of either (**A**) influenza virus A/PR/8/34 or (**B**) RSV B1. Cell lysates were generated from infected cells using CL Buffer supplemented with 1 mM DTT. Cell-lysate samples were subjected to one-step RT-qPCR targeting virus gene transcripts (influenza virus M1 or RSV N). The C_q values are plotted against $\log_{10}(TCID_{50})$; each point represents the mean with corresponding range among experimental replicates (n = 3).

component of a lysis buffer, may be a viable option for controlling RNases in cell lysates. However, we note the following. First, the cost associated with RNA*secure* (~$0.10 per 100 μL sample), while relatively modest compared with protein-based inhibitors of RNases, may nevertheless be deemed burdensome in high-throughput applications and avoidable in light of endogenous RI activity in cell lysates demonstrated in our study. Second, the heating step necessitated by RNA*secure* may be a logistical hindrance in high-throughput applications and likely inactivates endogenous RI in samples (*i.e.*, the modes of protection against RNases by RNA*secure* and endogenous RI are likely to be mutually exclusive). Following the heating step, cell-lysate samples containing RNA*secure* may be susceptible to contamination by adventitious RNases without the protection afforded by endogenous RI activity. Finally, the downstream impact of RNA*secure* on RT-qPCR has not been fully investigated, although successful application in this context has been reported [2].

Endogenous RI has been previously exploited for several applications. Systems for *in vitro* translation (rabbit reticulocyte lysate and mouse Krebs ascites) have been demonstrated to contain endogenous RI [13]; no improvement in translation efficiency was observed upon addition of exogenous RI to these systems. The high-speed supernatant fraction of rat liver tissue contains abundant endogenous RI [14], and this activity has been used to improve the efficiency of preparing rough microsomes from liver for *in vitro* translation and co-translational protein translocation [15]. Our study demonstrates that the utility of endogenous RI may not be restricted to applications deriving from enriched tissue sources (such as liver or placenta). On the basis of the known ubiquity of RI expression across cell types [9] as well as our own data (Figs. 3 and 4), we suggest that many (perhaps most) mammalian cell lines may contain an excess of endogenous RI activity that allows the

generation of cell lysates amenable to direct analysis by RT-qPCR without resorting to addition of exogenous RI, although end users ought to verify the suitability of this approach for their own cells of interest. In addition to the cell lines described in the present study (A549, HeLa, MDCK, and Vero), we have also successfully generated RT-qPCR-ready lysates using CL Buffer (with no exogenous RI) from MRC-5 human fibroblasts and ARPE-19 human retinal epithelial cells in our effort to develop a high-throughput microneutralization assay for human cytomegalovirus [16].

Addition of 1 mM DTT enhanced the maintenance of RNA integrity but was not sufficient to allow long-term frozen storage (>7 days) at −20 °C (Fig. 1A and B). We chose to include DTT in our routine CL Buffer formulation for many of our experiments. However, this decision may not be obligatory. One can choose to use freshly prepared DTT-free samples or to store DTT-free frozen samples at −80 °C; DTT-free samples frozen at −80 °C, once thawed, behave similarly to freshly prepared samples in terms of their RNA stability and resistance to thermal stress ([3] and unpublished data). The potential for inhibitory effects of DTT during downstream RT-qPCR has been reported [17], although we have not observed noticeable inhibition for the RT-qPCR used in the present study (data not shown). End users ought to explore the desirability of adding reducing agents to suit their particular experimental needs.

5. Conclusions

We have provided evidence that the preservation of RNA integrity in our crude cell lysates is due to the activity of endogenous RI. In addition, we have demonstrated the suitability of crude cell lysates prepared with our in-house cell-lysis buffer as direct input for downstream RT-qPCR. Use of crude cell-lysate samples can greatly facilitate RT-qPCR throughput by circumventing the need for RNA extraction. Concomitantly, avoidance of the need to add commercial RI to samples may also facilitate throughput by reducing per-sample cost. An understanding of the determinants of sample RNA stability might provide assurance regarding the quality of RT-qPCR data obtained with crude cell lysates and incentive for expanded use of this experimental approach. In addition, mechanistic awareness might encourage appropriate practices for sample handling (*i.e.*, in terms of sample storage temperature and requirement for reducing agents).

Subject categories

DNA recombinant techniques and nucleic acids.
RNA in all its forms.

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